Nucleic Acids Research, 2001, Vol. 29, No. 10 **2003–2011**

Transcription of the *Schizosaccharomyces pombe* U2 gene *in vivo* and *in vitro* is directed by two essential promoter elements

Dewang Zhou and Susan M. Lobo-Ruppert*

Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 844 BBRB, 845 19th Street South, Birmingham, AL 35294, USA

Received as resubmission February 26, 2001; Revised March 22, 2001; Accepted March 28, 2001

ABSTRACT

As compared to the metazoan small nuclear RNAs (snRNAs), relatively little is known about snRNA synthesis in unicellular organisms. We have analyzed the transcription of the *Schizosaccharomyces pombe* U2 snRNA gene *in vivo* and in the homologous *in vitro* system. Deletion and linker-scanning analyses show that the *S.pombe* U2 promoter contains at least two elements: the spUSE centered at –55, which functions as an activator, and a TATA box at –26, which is essential for basal transcription. These data point to a similar architecture among *S.pombe*, plant and invertebrate snRNA promoters. Factors recognizing the spUSE can be detected in whole cell extracts by DNase I footprinting and competition studies show that the binding of these factors correlates with transcriptional activity. Electrophoretic mobility shift assays and gel-filtration chromatography revealed a native molecular mass of ~200 kDa for the spUSE binding activity. Two polypeptides of molecular masses 25 and 65 kDa were purified by virtue of their ability to specifically bind the spUSE.

INTRODUCTION

In most organisms the majority of the spliceosomal small nuclear RNAs (snRNAs) are synthesized by RNA polymerase II (pol II), with the exception of the U6 snRNA, which is synthesized by RNA polymerase III (pol III) (reviewed in 1–3). The promoters of snRNA genes differ from those of other gene classes in several respects. First, snRNA genes usually contain transcriptional elements that are unique to this gene group (4,5). Secondly, the promoters of both RNA pol II and RNA pol III snRNA genes within a species are usually structurally related (6–10). Thirdly, although snRNA promoters are highly conserved within the same species, they vary greatly between different genera indicating that they have evolved rapidly (9–18).

Unlike the U6 gene, transcription of vertebrate pol II snRNA genes in cellular extracts has proven to be very difficult. Transcription of human U1 and U2 snRNA genes in HeLa cell extracts leads to synthesis not of mature snRNAs, but of large molecules aberrantly initiated upstream from the normal 5′-ends (19,20). Nevertheless, transient expression *in vivo* and the use of G-less cassette constructs *in vitro* showed that vertebrate snRNA promoters, including the U6 promoter, contain two major elements: a loosely conserved proximal sequence element (PSE) located at about position –55 relative to the transcription initiation site, and a well-conserved distal sequence element (DSE) located at around –200 (reviewed in 1–3). The PSE is a basal promoter element that defines the transcription start site and is the binding site for the factor SNAPc/PTF (21,22). The DSE functions as an enhancer and is required for maximal promoter activity. In addition to the PSE and DSE, the U6 gene promoter also contains an essential TATA box at –25 (7,8).

In contrast, the pol II snRNA genes of *Drosophila* (23), sea urchin (24,25), nematode (26) and dicotyledonous plants (27) can be accurately transcribed in homologous cell extracts. Characterization of snRNA promoters in these *in vitro* transcriptional systems as well as *in vivo* showed that in most cases they are bipartite. In *Drosophila*, both pol II and pol III snRNA gene promoters contain an essential 21 bp sequence element, called the PSEA, ~42 bp upstream of the transcription start site (17). The PSEB, a well-conserved 8 bp element, is also present at about –24 in the 5′ flanking region of pol II snRNA gene promoters. Instead of a PSEB sequence, the U6 promoter of *Drosophila* contains a canonical TATA box at a similar position (17). Surprisingly, the *Drosophila* U1 and U6 PSEAs cannot functionally substitute for each other; rather, the determination of RNA polymerase specificity is an intrinsic property of the PSEA sequences themselves (28). In the sea urchin, a sequence element termed the suPSE is located at about –55 upstream of the transcription start site in both pol II and pol III snRNA gene promoters (15,18,29). Despite the lack of sequence similarity between them, the U1 and U2 PSEs can substitute for the U6 PSE. Plant snRNA gene promoters consist of an upstream sequence element (USE) and a TATA box at –30 (10,12). The USE is a plant snRNA gene-specific enhancer and the spacing between the USE and TATA box is the major determinant of RNA polymerase specificity of plant snRNA genes (10,12).

snRNA gene expression in single-cell organisms is less well-characterized. Comparison of the 5′ flanking sequences of the

*To whom correspondence should be addressed. Tel: +1 205 975 8159; Fax: +1 205 975 7928; Email: sruppert@bmg125.cmc.uab.edu

U1 to U6 genes in fission yeast revealed the conservation of a TATA box 25 bp upstream of the transcription start site (30). One difference between pol II snRNA gene promoters (U1 to U5) and the pol III U6 promoter in this organism is that the sequences surrounding the TATA box in the pol II snRNA gene promoters are somewhat conserved, with a consensus sequence AtTTAcTATAWAYRY-RgGT (30). However, the functions of these sequences are unknown.

In this study we have investigated the transcription of *Schizosaccharomyces pombe* U2 snRNA gene *in vivo* and *in vitro*. Our results indicate that the *S.pombe* U2 promoter contains at least two essential elements: the *S.pombe* USE (spUSE) centered at –55 and a TATA box at –26. We have also characterized a protein complex that binds to the spUSE.

MATERIALS AND METHODS

Plasmid construction

Plasmid pSP-MU2, which contains the marked U2 gene, has been described previously (31). PCR was used to generate the U2 promoter deletion mutants (D1 to D6) with pSP-MU2 as the PCR template. The common 3′ primer used for PCR was: 5′-ACTGGATCCCCTCACATCCTAAACCATCCA-3′. The different 5′ oligonucleotides were as follows: U2D1, 5′-ACTGGATCCTCTGGTAACAAGTTTGTCAGC-3′; U2D2, 5′-ACTGGATCCGACCACAAAATTGGGACTGCGA-3′; U2D3, 5′-ACTGGATCCGACTGCGAAGTAATCTCAGG-3′; U2D4, 5′-ACTGGATCCTCAGGGTTCGGGTTTATTTATGAGA-3′; U2D5, 5′-ACAGGATCCATGAGATTTACTATATATACAGGT-3′; U2D6, 5′-ACTGGATCCTATATATACAGGTACTCGTG-3′. *Bam*HI sites are underlined. The PCR products were digested with *Bam*HI and cloned into the pSP1 vector (31). The sequences and orientation of the inserts were confirmed by DNA sequencing. Oligonucleotide-directed mutagenesis was performed using the method of Kunkel (32) to make U2 promoter linker-scanning mutants (PL1 to PL4). The sequences of the primers were as follows: U2PM1, 5′-TCGGGTTTATTTAT**TGTCGACGG**TATATATACAG-3′; U2PM2, 5′-TATTTATGAGATTTAC**GTCGACCCT**AGGTACT-3′; U2PM3, 5′-GATTTACTATATATAC**TGTCGACT**GTGCATTT-3′; U2PM4, 5′-TATACAGGTACTC**ACTTCGTCGACC**CGGTATTCTC-3′. The bold letters indicate the changed nucleotides in each mutant.

pUC-U6 was constructed by ligation of a 1.5 kb *Hin*cII fragment containing the *S.pombe* U6 gene (derived from pU6-5, a generous gift from Dr Y.Oshima) with pUC119. Plasmid ADH-U2 was prepared as follows. The DNA fragment containing the U2 coding region plus 64 nt of 3′ flanking sequences was obtained by PCR and cloned into pSP1. The resultant plasmid was named pSP-U2SAL. The oligonucleotides used for PCR were: U2SAL, 5′-TTCGTCGACCGGTATTCTCTCTTTGCCT-3′; SNU2-3′, 5′-ACGGGATCCCCTCACATCCTAAACCATCCA-3′. The *Sal*I and *Bam*HI sites are underlined. The ADH promoter was cut from plasmid pART3 (generously provided by Dr David Beach) with *Sal*I and cloned into the *Sal*I site of pSP-U2SAL. The orientation of the ADH promoter was confirmed by DNA sequencing.

Preparation of competitor DNA

For double-stranded spUSE competitor two oligonucleotides, D5F (5′-**CTAGT**TCAGGGTTCGGGTTTATTT**A**-3′) and D5R (5′-**CTAGT**AAATAAACCCGAACCCTGA**A**-3′) were annealed. The annealed fragment was then ligated with pSP1 linearized with *Spe*I. PCR, using T3 and T7 promoter primers complementary to vector sequences, was used to screen for clones containing inserts. The clone, pSP-D5, which contains two copies of spUSE was isolated and sequenced. To make specific competitor DNA, the 111 bp spUSE-containing *Eco*RI–*Sac*I fragment was excised from pSP-D5 and gel-purified. Double-stranded –20/+4 region competitors were also prepared in the same way. The two oligonucleotides used for cloning were: IF (5′-**CTAGT**CATTTGGTTCGGTATTCTCTCT**A**-3′) and IR (5′-**CTAGT**AGAGAGAATACCGAACCAAATG**A-**3′).

RNase T1 protection assay

The U2 promoter deletion mutants and the linker-scanning mutants were transformed into fission yeast strain SP130 (*h*$^{-}$ *ade6-M210 leu1-32*) and colonies were selected on minimal medium + adenine plates. Total RNAs were purified from *leu*$^{+}$ transformants and analyzed by RNase T1 protection assay as described previously (31). The antisense RNA probe used is complementary to the marked U2 RNA as shown in Figure 2A.

In vitro transcription

Schizosaccharomyces pombe whole cell extract preparation and *in vitro* transcription reactions were done as described (33,34), except that the transcription reactions were performed for 1 h at room temperature. In addition, 600 ng of template DNA, the optimal DNA concentration determined by titration, was used in each reaction. *In vitro* transcription was also performed in the presence of increasing amounts of spUSE and the –20/+4 oligonucleotides, or α-amanitin to test their effects on U2 transcription. Furthermore, the U6 snRNA gene and the ADH promoter were transcribed in the same extract in the presence of different concentrations of α-amanitin and used as controls to determine the RNA polymerase specificity of U2 gene transcription. PhosphorImager (Molecular Dynamics) analysis was used to quantitate the transcripts and the transcription of the mutants is expressed as a percentage of that derived from the wild-type promoter. Primer extension was performed to map the 5′-ends of the marked U2 transcripts as described previously (31). The oligonucleotide used for primer extension, U2PE (5′-AACTGAAAAGAACAGAG-3′), is complementary to bases 44–60 of the marked U2 gene.

DNase I footprinting

A DNA fragment extending from –120 upstream of the transcription start site to +60 in the coding region was prepared by PCR and used for DNase I footprinting. The primers used for PCR were U2D1 and U2PE described above. The DNA fragment was labeled with [γ-^{32}P]ATP by labeling one of the primers with T4 polynucleotide kinase before PCR. Aliquots of the same whole cell extract used for *in vitro* transcription reactions described above were used for DNase I footprinting. The DNase I footprinting assay was performed as described (35) except that all the steps were carried out at room temperature. Buffer Z′ with 100 mM KCl was used. The final products were

run on a 7% acrylamide/8 M urea sequencing gel. In addition, DNase I footprinting was also performed in the presence of increasing amounts of the pUSE or the –20/+4 competitors to test the sequence specificity of the DNase I footprint.

Electrophoretic mobility shift assay (EMSA)

The T7 primer was annealed to single-stranded DNA made from plasmid pSP-D5 and extended using Klenow fragment and [α-^{32}P]dCTP. The product was digested with *Eco*RI and *Not*I, and the resulting 90 bp spUSE fragment was then gel purified. For the binding assays 20 000 c.p.m. of the probe was incubated for 20 min at room temperature with either 1 µl of *S.pombe* whole cell extract or 9 µl of column fractions in a 30 µl reaction volume containing 50 mM HEPES pH 7.8, 90 mM potassium glutamate, 10 mM magnesium acetate, 5 mM EGTA, 10% glycerol, 0.75% PEG-3350 and 6 µg of poly(dI.dC) (Pharmacia). The DNA–protein complexes were resolved on native 4% polyacrylamide gels as described (36).

Gel-filtration chromatography

A 90 ml Sepharose 4B column was prepared and calibrated according to the instructions provided by the manufacturer (Bio-Rad). Gel-filtration chromatography was carried out in a buffer containing 20 mM HEPES pH 7.5, 20% glycerol, 10 mM $MgSO_4$, 10 mM EGTA and 5 mM DTT.

DNA affinity purification of spUSE binding proteins

The spUSE target DNA was obtained by PCR using an un-biotinylated T3 and 5′ biotinylated T7 promoter primers, and pSP-D5 as template. The control biotinylated DNA was obtained the same way except that insertless pSP1 vector was used as the PCR template. Biotinylated spUSE DNA or control DNA (1 µg) was incubated with 1.0 mg Dynabeads M-280 streptavidin (Dynal, Inc., Oslo, Norway) in 1× binding and washing buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA and 1 M NaCl) for 30 min at room temperature. After washing three times with 1× binding and washing buffer, the beads were re-suspended in 100 µl Buffer A (50 mM HEPES pH 7.5, 90 mM potassium glutamate, 10 mM magnesium acetate, 5 mM EGTA, 10% glycerol, 0.75% PEG-3350). Fraction 45 (320 µl; obtained by gel-filtration chromatography as described above) was incubated with 40 µl of DNA-coupled Dynabeads in 1× Buffer A for 30 min at room temperature. The beads were then washed three times with Buffer A containing 16 µg/ml poly(dI.dC). Finally, the bound proteins were eluted with 0.5 M NaCl, separated on a 12.5% SDS–polyacrylamide gel and visualized by silver staining.

RESULTS

Transcription of the *S.pombe* U2 snRNA gene is accurately initiated *in vitro*

A U2 snRNA gene marked by the introduction of a *Sac*I site between nucleotides 40 and 41 in the coding region (31) was used as a transcription template in an *S.pombe* whole cell extract. As shown in Figure 1A, an RNA band of the expected size (193 nt) was obtained in lane 2 but not in lane 1 in which pSP1 vector was used. We have previously shown that this marked U2 gene initiates transcription correctly *in vivo* (31). In order to confirm the identity of the RNA synthesized *in vitro*,

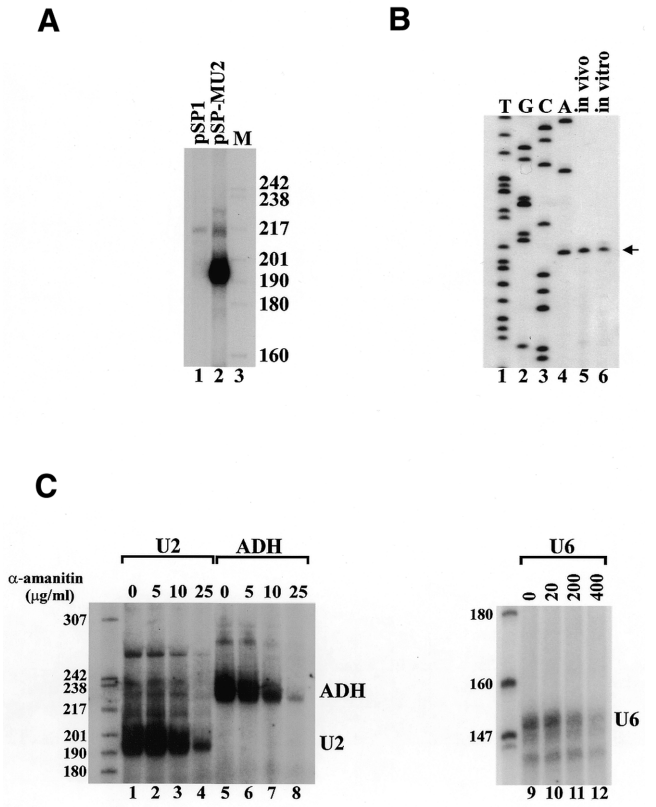

Figure 1. *In vitro* transcription of the *S.pombe* U2 snRNA gene. (**A**) *In vitro* transcription. An aliquot of 600 ng of pSP1 vector alone (lane 1) or plasmid pSP-MU2 (lane 2) were incubated with 3 µl of *S.pombe* whole cell extract as described (33,34) except that the transcription reaction was performed at room temperature for 1 h. The products were run on a 6% acrylamide/8 M urea sequencing gel. (**B**) Primer extension. A [γ-^{32}P]ATP 5′-labeled primer extending from nucleotide 60 to the *Sac*I site in the marked U2 gene, U2PE, was hybridized to 15 µg total RNA extracted from cells transformed with plasmid pSP-MU2 (lane 5) or RNA from one *in vitro* transcription reaction with plasmid pSP-MU2 (lane 6). The primer was extended with AMV reverse transcriptase, and the cDNA products were fractioned on a 6% acrylamide/8 M urea sequencing gel. The reference DNA sequence shown on the left was generated using the same labeled oligonucleotide. (**C**) α-Amanitin sensitivity of U2 transcription. Plasmids pSP-MU2, ADH-U2 and pUC-U6 were transcribed in whole cell extract in the presence of the indicated concentrations of α-amanitin. The sizes (in bp) of DNA standards are indicated to the side.

primer extension was performed using U2PE, an oligonucleotide complementary to the *Sac*I site at its 3′-end and AMV reverse transcriptase. The transcription initiation site was determined by electrophoresing the primer extension products adjacent to a DNA sequence ladder primed with the same end-labeled oligonucleotide (Fig. 1B). Our results indicate that the *in vitro* transcribed RNA is indeed U2 snRNA and that the marked U2 gene initiates transcription *in vitro* at the same A residue as *in vivo* (Fig. 1B, compare lane 6 with lane 5). As the U2 transcripts generated *in vitro* are of the expected length and are correctly initiated, it is likely that 3′-end formation also occurs accurately in the extract. To determine which RNA polymerase is utilized, we assayed transcription from the *S.pombe* U2 and ADH promoters in parallel in the same extract and in the presence of different concentrations of α-amanitin (Fig. 1C, left panel). U2 transcription is not detectably inhibited at 5 µg/ml of α-amanitin (Fig. 1C, compare lane 1 with lane 2) and is

reduced to 85.5 and 8.1% of control level in the presence of 10 and 25 μg/ml of α-amanitin, respectively (Fig. 1C, compare lanes 3 and 4 with lane 1). Transcription derived from the *S.pombe* ADH promoter has approximately the same α-amanitin sensitivity as that from the U2 promoter (Fig. 1C, lanes 5–8). In addition, we also transcribed the *S.pombe* U6 gene in the same whole cell extract in the presence of different concentrations of α-amanitin. As shown in the right panel of Figure 1C, 20 μg/ml of α-amanitin had no effect on U6 transcription (lane 10). At 200 μg/ml of α-amanitin, U6 transcription was only reduced to 60% of the level seen in the absence of inhibitor (Fig. 1C, compare lane 11 with lane 9). Twenty per cent of the control level of U6 transcription was observed even at 400 μg/ml of α-amanitin (Fig. 1C, lane 12), correlating well with published data showing that *S.pombe* RNA pol III is more resistant to this inhibitor than the corresponding mammalian enzyme (37).

Sequences upstream of the TATA box are required for U2 gene transcription *in vivo* and *in vitro*

A series of U2 promoter deletion mutants was made (Fig. 2A) to determine the minimal region required for transcription. As shown in Figure 2B, deletion of the U2 promoter to –120 had no effect on transcription *in vitro* (compare lane 3 with lane 2), while deletion to –92 reduced the transcription level to only 50% of the wild-type level (compare lane 4 with lane 2). Further deletion of the U2 promoter to –78 and –63 did not reduce U2 transcription further (Fig. 2B, compare lanes 5 and 6 with lane 4) suggesting that only the sequences between –120 and –92 had some effect on U2 transcription *in vitro.* Deletion of the U2 promoter to –44 further reduced U2 transcription to only 2% of the wild-type level (Fig. 2B, compare lane 7 with lane 2). This result indicates that the sequence between –63 and –44 is required for maintenance of a high level of U2 transcription. Finally, deletion of the U2 promoter to –34, 1 nt upstream of the TATA box, abolished U2 transcription completely (Fig. 2B, lane 8) indicating that the TATA box alone is not sufficient to direct U2 transcription *in vitro* and that upstream sequences are required.

We next transformed the wild-type U2 promoter construct and the U2 promoter deletion mutants into a *leu⁻ S.pombe* strain (SP130). Total RNAs were extracted from *leu*$^+$ transformants and analyzed by RNase T1 protection of the antisense RNA probe shown in Figure 2A. In this experiment the antisense RNA probe was in excess. The copy number of the pSP1 vector is essentially constant as RNAs isolated from either single colonies or from pools of 10 colonies gave the same results when assayed in parallel (data not shown). The endogenous U2 snRNA (Fig. 2C, e-U2) serves as an internal control. In contrast to the *in vitro* system, deletion of the U2 promoter to position –63 had no effect on U2 transcription *in vivo* (Fig. 2C, lanes 4–7). As was seen *in vitro* (Fig. 2B), further deletion of the U2 promoter to position –44 reduced U2 transcription to only 6.5% of the wild-type level *in vivo* (Fig. 2C, lane 8); and deletion of U2 promoter to –34 further debilitated, but did not completely abrogate, U2 transcription (Fig. 2C, lane 9). In summary, the sequence between positions –63 and –44 is required for high level U2 expression *in vivo* and *in vitro*. We refer to this element as the spUSE. This result differs from an earlier report, which showed that the sequences upstream of the TATA box were not required (38).

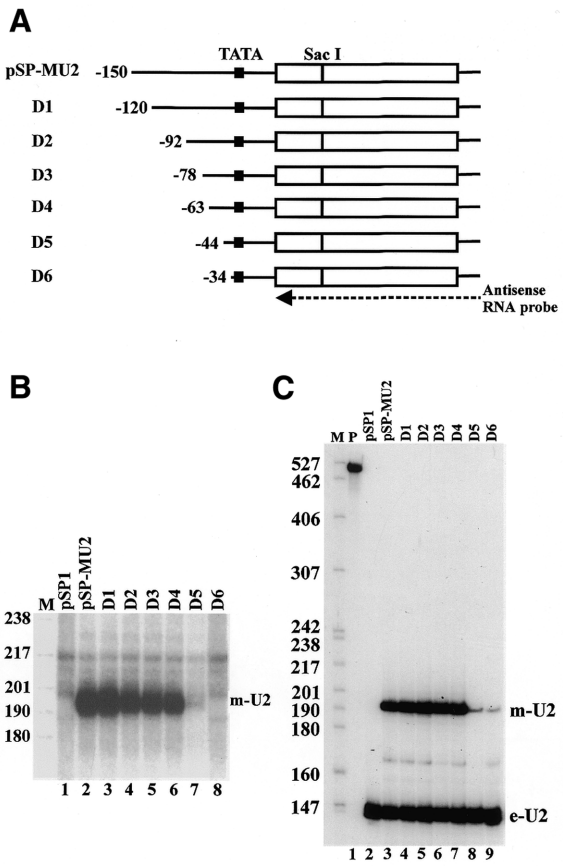

Figure 2. Sequences upstream of the TATA box are required for U2 transcription. (**A**) Diagram of the U2 promoter deletion mutants. The U2 gene was marked by introducing a *Sac*I site between nucleotides 40 and 41 in the coding region. (**B**) *In vitro* transcription. Plasmid pSP-MU2 and the U2 promoter deletion mutants were transcribed in the whole cell extract and the products were run on a 6% acrylamide/8 M urea sequencing gel. (**C**) RNase T1 protection assay of U2 snRNAs. Plasmid pSP-MU2 and the U2 promoter deletion mutants were transformed into fission yeast strain SP130. Total RNAs were purified from *leu*$^+$ transformants. The antisense RNA probe (in excess) shown in Figure 2A was used. The RNase T1 protection assay was performed as described previously (31). (B and C) The sizes of DNA standards (in bp; lane M) are indicated on the left.

Sequences surrounding the TATA box affect U2 transcription *in vitro*

Comparison of the *S.pombe* U1 to U5 pol II snRNA gene promoters showed that the sequences surrounding the TATA box in these promoters are somewhat conserved (30,38). However, the significance of this conservation was not clear nor was it known if sequences downstream of the TATA box were essential for transcription. Results shown in Figure 2 suggest that the sequences immediately upstream of the TATA box may contribute to the basal level of transcription. In order to examine the function of the sequences surrounding the TATA box in more detail we made the series of linker-scanning mutations shown in Figure 3A and tested their effect on U2 transcription *in vitro* and *in vivo* (Fig. 3B and C). *In vitro*, the TATA box is essential as mutation of this sequence abolished U2 transcription (Fig. 3B, lane 3). Sequences immediately upstream and downstream of the TATA box are also required as mutation of these sequences reduced U2 transcription to ~48

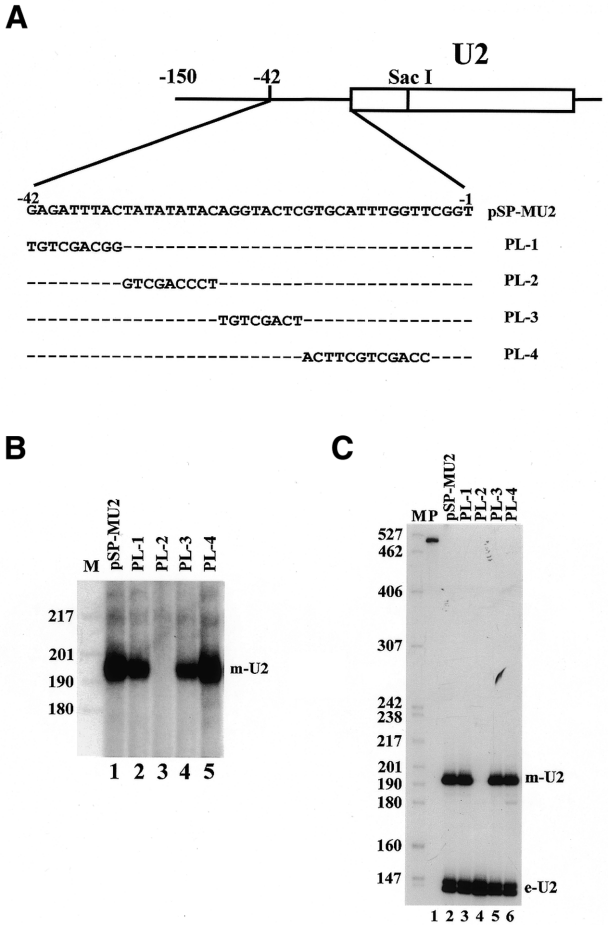

Figure 3. Role of the TATA box and adjacent sequences on U2 transcription. (**A**) Diagram of the U2 promoter linker-scanning mutants. (**B**) *In vitro* transcription. Plasmid pSP-MU2 and the U2 promoter linker-scanning mutants were transcribed in whole cell extract and the products were run on a 6% acrylamide/8 M urea sequencing gel. (**C**) RNase T1 protection assay of U2 snRNAs. The U2 promoter linker-scanning mutants were transformed into fission yeast strain SP130. Total RNAs were purified and subjected to RNase T1 protection assay as in Figure 2C. The antisense RNA probe was in excess. (B and C) The size (in bp) of DNA standards are indicated on the left.

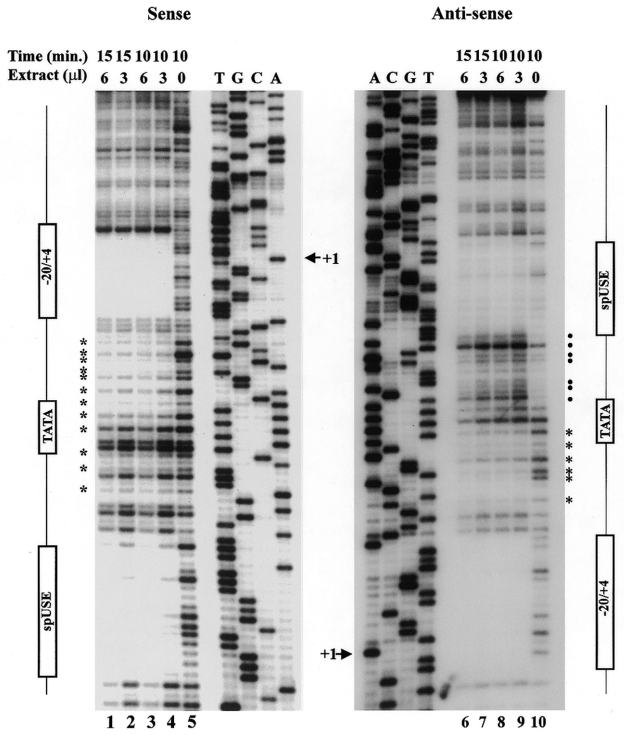

Figure 4. DNase I footprinting of the U2 promoter. (Left) DNase I footprinting of the sense strand. U2D1 was labeled with [γ-^{32}P]ATP. The DNA sequence ladders were prepared using the same labeled primer. (Right) DNase I footprinting of the antisense strand. U2PE was labeled with [γ-^{32}P]ATP. The DNA sequence ladders were prepared with the labeled U2PE. For DNase I footprinting 50 000 c.p.m. of the probe was used in each sample. The amounts of *S.pombe* whole cell extract used and the incubation time of the DNA probe with the cell extract at room temperature before addition of DNase I are indicated above the lanes. Positions that are partially protected from DNase I digestion are indicated by asterisks and positions of enhanced cleavage are indicated by dots.

or 45% of the wild-type level (Fig. 3B, compare lanes 2 and 4 with lane 1). Mutation of the sequence upstream of position –4 increased transcription by 50% over that of the wild-type U2 promoter (Fig. 3B, compare lane 5 with lane 1). We next used RNase T1 protection of the antisense RNA probe shown in Figure 2A to measure the steady-state levels of transcripts derived from the linker-scanning mutants *in vivo*. Again, the antisense RNA probe was in excess. As shown in Figure 3C, mutation of the TATA box abolished U2 transcription (Fig. 3C, lane 4) whereas mutation of the sequences surrounding the TATA box or between –20 and –4 upstream of the transcription initiation site had no effect on U2 expression (Fig. 3C, lanes 3, 5 and 6). Primer extension analysis of RNAs derived from transcription of these linker-scanning mutants *in vivo* and *in vitro* (data not shown) indicates that they initiate at the same nucleotide as in the wild-type construct. Thus, the TATA box is essential for U2 transcription *in vivo* and *in vitro*.

The binding of proteins to the U2 promoter correlates with transcription *in vitro*

DNase I footprinting was used to identify the binding sites of proteins from a transcriptionally active whole cell extract on the U2 promoter. A probe extending from –120 upstream of the transcription start site to +60 in the coding region was generated by PCR using a 5′-end-labeled oligonucleotide, and was incubated with whole cell extract and treated with DNase I. As shown in Figure 4 (left panel), the sequence between –63 and –44 (spUSE) on the sense strand is protected from DNase I digestion in the presence of a whole cell *S.pombe* extract. In addition, the sequences around the transcription initiation site are also protected. We refer to these sequences as the –20/+4 element. In contrast, there is only partial protection of certain nucleotides (Fig. 4, asterisks) within, and adjacent to, the TATA box sequence. This weak footprint is similar to those seen on the TATA boxes of mRNA gene promoters from higher eukaryotes (39). It may result from binding of a factor and/or distortion of the DNA helix. On the antisense strand, as on the sense strand, strong protection was seen on the –20/+4 region and the spUSE, with partial protection of the TATA box and sequences between the TATA box and the –20/+4 region

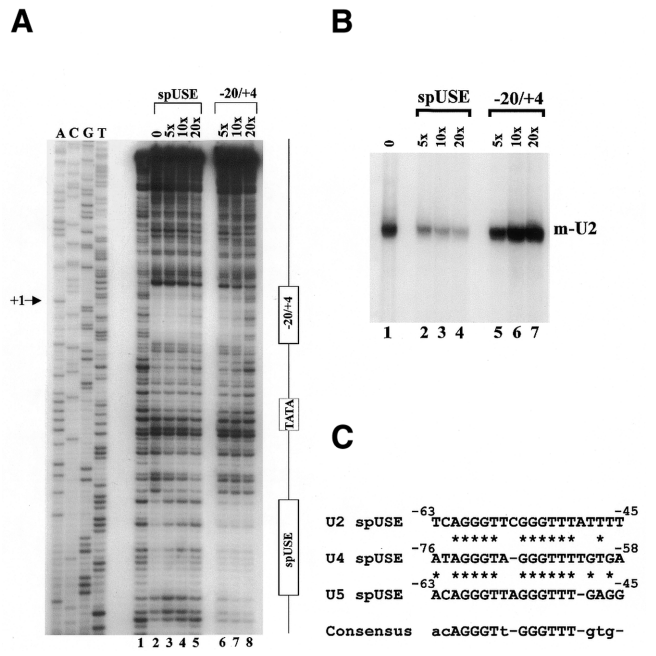

Figure 5. The binding of proteins to the spUSE and –20/+4 region correlates with U2 transcription. (**A**) The effect of spUSE and –20/+4 competitors on DNase I footprinting on the sense strand of the U2 promoter. The DNase I footprinting assays were performed as in Figure 4. The amounts of spUSE and –20/+4 competitors used relative to the radiolabeled probe are indicated. (**B**) The effect of spUSE and –20/+4 competitors on U2 transcription. Plasmid pSP-MU2 was transcribed in the presence of increasing amounts of spUSE and –20/+4 competitors. (**C**) Comparison of spUSE-like sequences in the *S.pombe* U2, U4 and U5 snRNA gene promoters. In the consensus sequence, nucleotides conserved in all three promoters are shown in upper case, while lower case letters indicate nucleotides conserved in two of the three promoters.

(Fig. 4, right panel, asterisks). In addition, there is enhanced DNase I cleavage of the sequences between the TATA box and spUSE (Fig. 4, dots). We then performed DNase I footprinting analysis in the presence of double-stranded DNA competitors derived from the spUSE or from the –20/+4 region to test the specificity of protein binding. The results are shown in Figure 5A. The DNase I footprint between –63 and –44 became weaker as the amount of spUSE was increased whereas the footprint around the transcription initiation site was unaffected (Fig. 5A, lanes 3–5). Similarly, the DNase I footprint around the transcription initiation site was replaced with increasing amounts of the –20/+4 competitor DNA whereas the DNase I footprint between –63 and –44 was not (Fig. 5A, lanes 6–8). As there is no cross-competition, these data suggest that different factors bind to the spUSE and the –20/+4 region and that the binding is sequence-specific.

To determine if protein binding to spUSE and the –20/+4 region correlates with transcription, we transcribed the U2 gene *in vitro* in the presence of increasing amounts of spUSE or –20/+4 competitors. As shown in Figure 5B, transcription of the U2 gene was reduced dramatically with increasing amounts of spUSE (compare lanes 2–4 with lane 1). A 5-fold excess of spUSE competitor reduces U2 transcription to 30% of the level seen in the absence of competitor (Fig. 5B, compare lane 2 with lane 1) and a 20-fold excess of spUSE competitor further reduces transcription to 13% (Fig. 5B, compare lane 4 with lane 1). Higher concentrations of spUSE did not reduce U2 transcription further (data not shown) suggesting that spUSE may function as an activator element. In contrast, transcription increases as the amount of the –20/+4 region competitor is increased (Fig. 5B, compare lanes 5–7 with lane 1) suggesting that the corresponding protein(s) may function as a repressor. These results are consistent with the effects of the linker-scanning mutations on *in vitro* transcription, as shown in Figure 3B, and correlate well with the competition of the DNase I footprints shown in Figure 5A. Nevertheless, mutation of part of the –20/+4 sequence has no detectable effect on U2 transcription *in vivo* (Fig. 3C).

Characterization of a transcription factor that binds to the spUSE element

We next used the spUSE as a probe in EMSAs. Using an *S.pombe* whole cell extract, at least four protein–DNA complexes (a–d) were observed (Fig. 6A, lane 2). Addition of competitor oligonucleotides demonstrated the sequence specificity of these interactions, since an excess of the unlabeled spUSE competed for complex formation (Fig. 6A, lanes 3 and 4) while addition of non-specific DNA oligonucleotides did not (Fig. 6A, lanes 5 and 6). The appearance of multiple protein–DNA complexes in the EMSA suggests that the protein binding activity of spUSE may be a multi-subunit protein complex that partially dissociates during electrophoresis. The overall mass of this complex was estimated by gel-filtration chromatography (Fig. 6B). The spUSE binding activity, as determined by the EMSA, peaked at fraction 45 on a Sephadex 4B column, indicating a molecular weight of ~200 kDa for this complex. Magnetic streptavidin beads (Dynabeads, M280-streptavidin; Dynal, Inc.) coupled with biotinylated spUSE were used to purify the proteins that interact with the spUSE. As shown in Figure 6C, two proteins with molecular masses of 25 and 65 kDa were detected by SDS–PAGE and silver staining, respectively (Fig. 6C, lane 2). The specificity of binding of these two proteins was demonstrated by their absence in lane 3 (Fig. 6C), where an excess of un-biotinylated spUSE competitor was added, and in lane 1 (Fig. 6C), where only unrelated vector DNA was coupled to the magnetic beads. Our data suggests that the transcription factor that binds to the spUSE contains at least two different proteins.

DISCUSSION

Our results demonstrate that, like the sea urchin U2 (15,18,29) and plant snRNA gene promoters (10,12), the *S.pombe* U2 snRNA promoter is bipartite. It contains at least two essential promoter elements: the spUSE centered at –55 and a TATA box at –26. The TATA box is a basal element as mutation of this sequence abolishes transcription *in vivo* and *in vitro* (Fig. 3B and C). In contrast, the spUSE functions as an activator because its deletion reduces U2 transcription *in vivo* and *in vitro* (Fig. 2B and C). Our results differ from those reported in an earlier study of U2 snRNA gene expression in fission yeast, which showed that the sequences upstream of the TATA box were not required (38). These researchers relied on a primer extension assay using an oligonucleotide that differed from the wild-type U2 sequence at a single nucleotide at its 3′-end to measure transcription from an exogenous U2 gene marked by a single nucleotide insertion. It is possible that this assay did

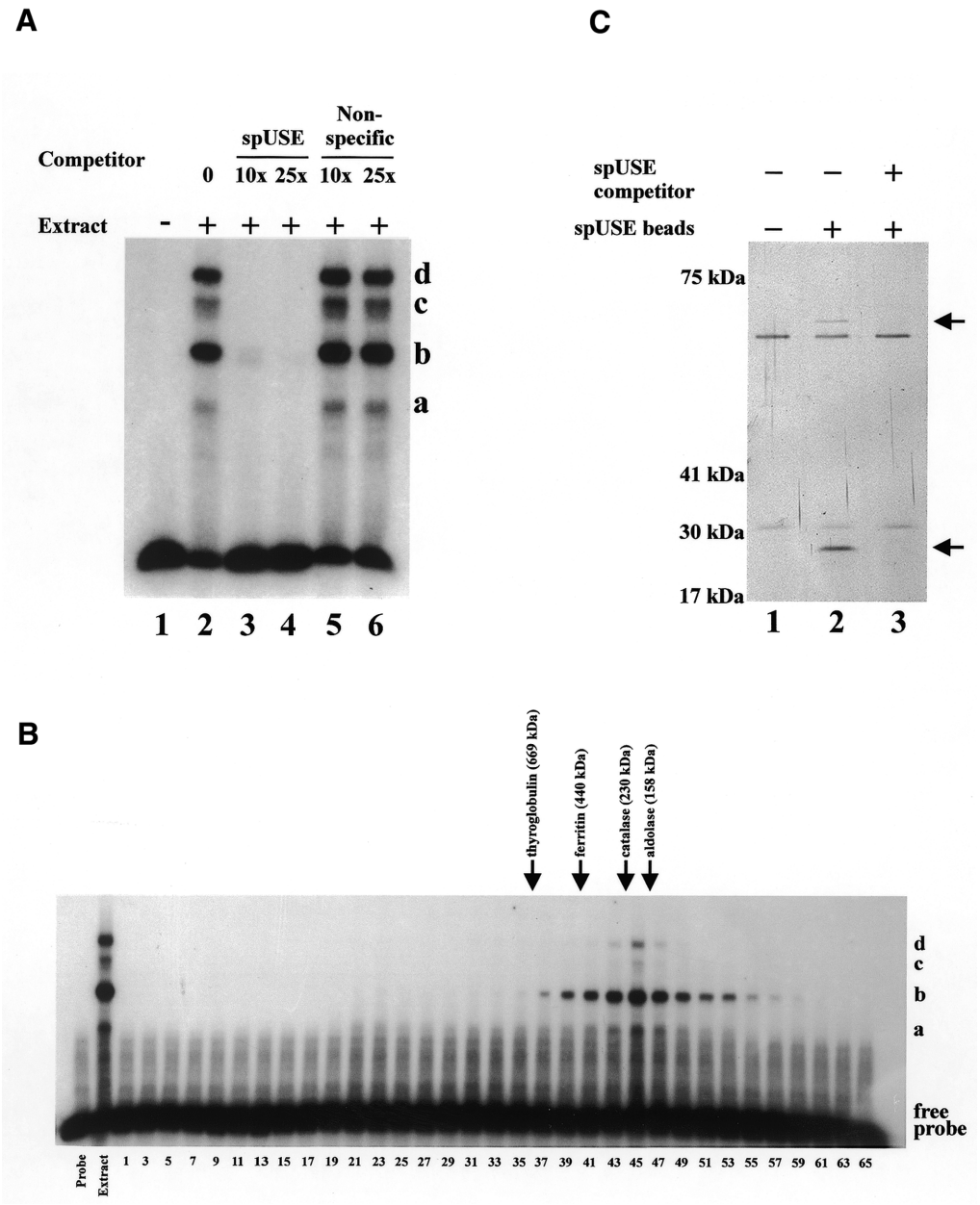

Figure 6. Characterization of spUSE binding activity in an *S.pombe* whole cell extract. (**A**) A double-stranded, radiolabeled spUSE probe was incubated with extract and analyzed by EMSA as detailed in Materials and Methods. For each sample, 20 000 c.p.m. (4 fmol) of the probe was incubated with 50 µg (1 µl) of *S.pombe* whole cell extract. Unlabeled oligonucleotide competitors were added to the extract protein in the fold-molar excess indicated (10×, 40 fmol; 25×, 100 fmol). In lanes 3 and 4, unlabeled spUSE competitor was added. In lanes 5 and 6, a vector DNA fragment of approximately the same size was used as non-specific competitor. (**B**) Estimate of the molecular weight of the spUSE binding factor by gel-filtration chromatography. One milliliter of *S.pombe* whole cell extract was loaded on a calibrated Sepharose 4B column (90 ml). The positions of elution of molecular weight standards are indicated at the top. Fractions were analyzed for spUSE binding activity by EMSA as in (A). The peak of the spUSE binding activity was eluted in fraction 45. (**C**) Purification of proteins that bind to the spUSE. The biotinylated spUSE and control DNAs were coupled to Dynabeads M280-streptavidin beads, incubated with fraction 45 and analyzed as described in Materials and Methods. In lane 3, 5 µg (~12-fold molar excess) of unbiotinylated spUSE was added.

not completely distinguish between transcripts derived from the endogenous and exogenous U2 genes.

Examination of the *S.pombe* U4 and U5 promoter sequences shows that spUSE-like elements also exist in these promoters at similar positions (Fig. 5C). Four complexes, a–d, are observed to form on the U2 spUSE in EMSAs (Fig. 6A). These complexes co-migrate during Sephadex 4B chromatography and elute at an apparent molecular weight of ~200 kDa (Fig. 6B). The peak fraction from the Sephadex 4B column contains two polypeptides of 25 and 65 kDa that specifically bind to the spUSE (Fig. 6C). As the combined molecular mass of these peptides is approximately half that estimated for the spUSE factor by gel-filtration chromatography, it is possible that they may bind as a heterotetramer containing two copies of each

peptide. The spUSE sequence consists of two imperfect direct repeats and we speculate that each repeat may constitute a half-site that binds one copy of the 25 and 65 kDa peptides.

Our analysis of the *S.pombe* U2 promoter indicates that the sequences surrounding the TATA box contribute to transcription *in vitro*. The sequences immediately upstream and downstream of the TATA box show either enhanced DNase I cleavage or partial protection from cleavage when proteins from the homologous extract are bound to the promoter (Fig. 4), indicating that they may contribute to the formation or stability of the transcription initiation complex. However, mutation of these sequences has little effect on the level of U2 snRNA *in vivo* (Figs 2C and 3C). As these sequences are semi-conserved in the fission yeast U1 to U5 snRNA promoters but not in the U6 promoter (30,38), it is possible that they have an *in vivo* function that was not detected in our assay.

A concentration of >25 μg/ml of α-amanitin is required to completely inhibit transcription from the *S.pombe* U2 and ADH promoters *in vitro* (Fig. 1C). It was previously reported that transcription by *S.pombe* pol II was abrogated by 10 μg/ml of this inhibitor (40,41). However, in these experiments the templates used were heterologous. Woontner and Jaehning. (40) used a chimera composed of the *Saccharomyces cerevisiae* GAL4 UAS, the *S.cerevisiae* CYC1 TATA box and initiation region, fused to a G-less cassette. Flanagan *et al*. (41) used the Adenovirus 2 Major Late promoter fused to a G-less cassette. In both cases, transcription levels appear to have been lower than those observed by us from the *S.pombe* U2 and ADH promoters. Thus, where we observe a 15% reduction in U2 and ADH promoter derived transcription at 10 μg/ml of α-amanitin, it is likely that the level of transcription observed by these workers was too low to be detectable. In contrast to *S.pombe*, in higher eukaryotes complete inhibition of RNA pol II transcription occurs at much lower concentrations (2–10 μg/ml) of α-amanitin (42). The largest subunit of *S.pombe* RNA pol II contains a substitution of the conserved Asn residue at position 775 of domain F by Ile (43). This residue and its neighbors in domain F are highly conserved among higher eukaryotes. An α-amanitin resistant mutant of mouse RNA pol II contains an Asn to Asp mutation in domain F (44). Furthermore, in the largest subunit of *S.cerevisiae* pol II there is a Ser residue at position 769, which corresponds to Ile 775 in the *S.pombe* homolog. As suggested by Azuma *et al*. (43), these substitutions may account for the higher resistance of the fungal RNA pol II enzymes to α-amanitin (this work, 45). *Schizosaccharomyces pombe* RNA pol III is also less sensitive to α-amanitin than the corresponding enzyme from metazoans. Using α-amanitin at a concentration of 200 μg/ml is usually sufficient to completely inhibit RNA pol III in higher eukaryotes (42,46,47); in contrast, at this concentration, *S.pombe* U6 transcription is barely affected (Fig. 1C). It was recently shown that fairly high levels of 7SL transcription by *S.pombe* RNA pol III are observed even in the presence of 1 mg/ml α-amanitin (37). These results suggest that in *S.pombe* both RNA pol II and RNA pol III are more robust to α-amanitin than the corresponding metazoan enzymes.

As the *S.pombe* U2 snRNA gene is accurately transcribed *in vitro* the powerful combination of biochemical and genetic techniques can be brought to bear on studying the mechanisms involved in the synthesis of this interesting class of RNAs.

ACKNOWLEDGEMENTS

We thank Dr Y.Oshima for the *S.pombe* U6 gene, Dr D.Beach for the pART3 vector and Sabrina Walthall for assistance with chromatography. This work was supported by NIH grant RO1GM51908 to S.M.L.-R.

REFERENCES

1. Dahlberg,J.E. and Lund,E. (1988). The genes and transcription of the major small nuclear RNAs. In Berstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles.* Springer-Verlag, Berlin, Germany, pp. 38–70.
2. Hernandez,N., Lobo,S., Lister,J., Ifill,S., Ratnasabapathy,R., Sheldon,M. and Johal,L. (1990) Transcription of the human U2 and U6 RNA genes. *Mol. Biol. Rep.*, **14**, 167.
3. Lobo,S.M. and Hernandez,N. (1994) *Transcription of snRNA Genes by RNA Polymerases II and III*. Raven Press, New York, NY.
4. Skuzeski,J.M., Lund,E., Murphy,J.T., Steinberg,T.H., Burgess,R.R. and Dahlberg,J.E. (1984) Synthesis of human U1 RNA. II. Identification of two regions of the promoter essential for transcription initiation at position +1. *J. Biol. Chem.*, **259**, 8345–8352.
5. Tanaka,M., Grossniklaus,U., Herr,W. and Hernandez,N. (1988) Activation of the U2 snRNA promoter by the octamer motif defines a new class of RNA polymerase II enhancer elements. *Genes Dev.*, **2**, 1764–1778.
6. Carbon,P., Murgo,S., Ebel,J.P., Krol,A., Tebb,G. and Mattaj,L.W. (1987) A common octamer motif binding protein is involved in the transcription of U6 snRNA by RNA polymerase III and U2 snRNA by RNA polymerase II. *Cell*, **51**, 71–79.
7. Mattaj,I.W., Dathan,N.A., Parry,H.D., Carbon,P. and Krol,A. (1988) Changing the RNA polymerase specificity of U snRNA gene promoters. *Cell*, **55**, 435–442.
8. Lobo,S.M. and Hernandez,N. (1989) A 7 bp mutation converts a human RNA polymerase II snRNA promoter into an RNA polymerase III promoter. *Cell*, **58**, 55–67.
9. Waibel,F. and Filipowicz,W. (1990) RNA-polymerase specificity of transcription of *Arabidopsis* U snRNA genes determined by promoter element spacing. *Nature*, **346**, 199–202.
10. Waibel,F. and Filipowicz,W. (1990) U6 snRNA genes of *Arabidopsis* are transcribed by RNA polymerase III but contain the same two upstream promoter elements as RNA polymerase II- transcribed U-snRNA genes. *Nucleic Acids Res.*, **18**, 3451–3458.
11. Lo,P.C. and Mount,S.M. (1990) *Drosophila melanogaster* genes for U1 snRNA variants and their expression during development. *Nucleic Acids Res.*, **18**, 6971–6979.
12. Vankan,P. and Filipowicz,W. (1989) A U-snRNA gene-specific upstream element and a –30 'TATA box' are required for transcription of the U2 snRNA gene of *Arabidopsis thaliana*. *EMBO J.*, **8**, 3875–3882.
13. Murgo,S., Krol,A. and Carbon,P. (1991) Sequence, organization and transcriptional analysis of a gene encoding a U1 snRNA from the axolotl, *Ambystoma mexicanum*. *Gene*, **99**, 163–170.
14. Simmen,K.A., Waldschmidt,R., Bernues,J., Parry,H.D., Seifart,K.H. and Mattaj,I.W. (1992) Proximal sequence element factor binding and species specificity in vertebrate U6 snRNA promoters. *J. Mol. Biol.*, **223**, 873–884.
15. Stefanovic,B. and Marzluff,W.F. (1992) Characterization of two developmentally regulated sea urchin U2 small nuclear RNA promoters: a common required TATA sequence and independent proximal and distal elements. *Mol. Cell. Biol.*, **12**, 650–660.
16. Thomas,J., Lea,K., Zucker-Aprison,E. and Blumenthal,T. (1990) The spliceosomal snRNAs of *Caenorhabditis elegans*. *Nucleic Acids Res.*, **18**, 2633–2642.
17. Zamrod,Z., Tyree,C.M., Song,Y. and Stumph,W.E. (1993) *In vitro* transcription of a *Drosophila* U1 small nuclear RNA gene requires TATA box-binding protein and two proximal *cis*-acting elements with stringent spacing requirements. *Mol. Cell. Biol.*, **13**, 5918–5927.
18. Wendelburg,B.J. and Marzluff,W.F. (1992) Two promoter elements are necessary and sufficient for expression of the sea urchin U1 snRNA gene. *Nucleic Acids Res.*, **20**, 3743–3751.
19. Murphy,J.T., Burgess,R.R., Dahlberg,J.E. and Lund,E. (1982) Transcription of a gene for human U1 small nuclear RNA. *Cell*, **29**, 265–274.
20. Westin,G., Lund,E., Murphy,J.T., Pettersson,U. and Dahlberg,J.E. (1984) Human U2 and U1 RNA genes use similar transcription signals. *EMBO J.*, **3**, 3295–3301.

21. Henry,R.W., Sadowski,C.L., Kobayashi,R. and Hernandez,N. (1995) A TBP-TAF complex required for transcription of human snRNA genes by RNA polymerase II and III. *Nature*, **374**, 653–656.
22. Yoon,J.B., Murphy,S., Bai,L., Wang,Z. and Roeder,R.G. (1995) Proximal sequence element-binding transcription factor (PTF) is a multisubunit complex required for transcription of both RNA polymerase II- and RNA polymerase III-dependent small nuclear RNA genes. *Mol. Cell. Biol.*, **15**, 2019–2027.
23. Kamakaka,R.T., Tyree,C.M. and Kadonaga,J.T. (1991) Accurate and efficient RNA polymerase II transcription with a soluble nuclear fraction derived from *Drosophila* embryos. *Proc. Natl Acad. Sci. USA*, **88**, 1024–1028.
24. Morris,G.F. and Marzluff,W.F. (1985) Synthesis of U1 RNA in isolated nuclei from sea urchin embryos: U1 RNA is initiated at the first nucleotide of the RNA. *Mol. Cell. Biol.*, **5**, 1143–1150.
25. Morris,G.F., Price,D.H. and Marzluff,W.F. (1986) Synthesis of U1 RNA in a DNA-dependent system from sea urchin embryos. *Proc. Natl Acad. Sci. USA*, **83**, 3674–3678.
26. Hannon,G.E., Hannon,G.J., Maroney,P.A. and Nilsen,T.W. (1994) Transcription of a nematode U1 small nuclear RNA *in vitro*. 3′-End formation requires *cis*-acting elements within the coding sequence. *J. Biol. Chem.*, **269**, 12387–12390.
27. Filipowicz,W., Kiss,T., Marshallsay,C. and Waibel,F. (1990) U-snRNA genes, U-snRNAs and U-snRNPs of higher plants. *Mol. Biol. Rep.*, **14**, 125–129.
28. Jensen,R.C., Wang,Y., Hardin,S.B. and Stumph,W.E. (1998) The proximal sequence element (PSE) plays a major role in establishing the RNA polymerase specificity of *Drosophila* U-snRNA genes. *Nucleic Acids Res.*, **26**, 616–622.
29. Li,J.M., Parsons,R.A. and Marzluff,W.F. (1994) Transcription of the sea urchin U6 gene *in vitro* requires a TATA-like box, a proximal sequence element, and sea urchin USF, which binds an essential E box. *Mol. Cell. Biol.*, **14**, 2191–2200.
30. Porter,G., Brennwald,P. and Wise,J.A. (1990) U1 small nuclear RNA from *Schizosaccharomyces pombe* has unique and conserved features and is encoded by an essential single-copy gene. *Mol. Cell. Biol.*, **10**, 2874–2881.
31. Zhou,D., Frendewey,D. and Lobo-Ruppert,S.M. (1999) Pac1p, an RNase III homolog, is required for formation of the 3′ end of U2 snRNA in *Schizosaccharomyces pombe*. *RNA*, **5**, 1083–1098.
32. Kunkel,T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
33. Woontner,M. and Jaehning,J.A. (1990) Accurate initiation by RNA polymerase II in a whole cell extract from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 8979–8982.
34. Woontner,M., Wade,P.A., Bonner,J. and Jaehning,J.A. (1991) Transcriptional activation in an improved whole-cell extract from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 4555–4560.
35. Schmitz,A. and Galas,D.J. (1980) Sequence-specific interactions of the tight-binding I12-X86 lac repressor with non-operator DNA. *Nucleic Acids Res.*, **8**, 487–506.
36. Lobo-Ruppert,S.M., McCulloch,V., Meyer,M., Bautista,C., Falkowski,M., Stunnenberg,H.G. and Hernandez,N. (1996) Monoclonal antibodies directed against the amino-terminal domain of human TBP cross-react with TBP from other species. *Hybridoma*, **15**, 55–68.
37. Rodicker,F., Ossenbuhl,F., Michels,D. and Benecke,B.J. (1999) Faithful *in vitro* transcription by fission yeast RNA polymerase III reveals unique α-amanitin sensitivity. *Gene Expr.*, **8**, 165–174.
38. Dandekar,T. and Tollervey,D. (1991) Thirty-three nucleotides of 5′ flanking sequence including the 'TATA' box are necessary and sufficient for efficient U2 snRNA transcription in *Schizosaccharomyces pombe*. *Mol. Microbiol.*, **5**, 1621–1625.
39. Schmidt,M.C., Zhou,Q. and Berk,A.J. (1989) Sp1 activates transcription without enhancing DNA-binding activity of the TATA box factor. *Mol. Cell. Biol.*, **9**, 3299–3307.
40. Woontner,M. and Jaehning,J.A. (1993) Accurate initiation of mRNA synthesis in extracts from *Schizosaccharomyces pombe*, *Kluyveromyces lactis* and *Candida glabrata*. *Yeast*, **9**, 1331–1334.
41. Flanagan,P.M., Kelleher,R.J., Feaver,W.J., Lue,N.F., LaPointe,J.W. and Kornberg,R.D. (1990) Resolution of factors required for the initiation of transcription by yeast RNA polymerase II. *J. Biol. Chem.*, **265**, 11105–11107.
42. Chambon,P. (1975) Eukaryotic nuclear RNA polymerases. *Annu. Rev. Biochem.*, **44**, 613–638.
43. Azuma,Y., Yamagishi,M., Ueshima,R. and Ishihama,A. (1991) Cloning and sequence determination of the *Schizosaccharomyces pombe rpb1* gene encoding the largest subunit of RNA polymerase II. *Nucleic Acids Res.*, **19**, 461–468.
44. Bartolomei,M.S. and Corden,J.L. (1987) Localization of an α-amanitin resistance mutation in the gene encoding the largest subunit of mouse RNA polymerase II. *Mol. Cell. Biol.*, **7**, 586–594.
45. Schultz,L.D. and Hall,B.D. (1976) Transcription in yeast: α-amanitin sensitivity and other properties which distinguish between RNA polymerases I and III. *Proc. Natl Acad. Sci. USA*, **73**, 1029–1033.
46. Huet,J., Manaud,N., Dieci,G., Peyroche,G., Conesa,C., Lefebvre,O., Ruet,A., Riva,M. and Sentenac,A. (1996) RNA polymerase III and class III transcription factors from *Saccharomyces cerevisiae*. *Methods Enzymol.*, **273**, 249–267.
47. Klekamp,M.S. and Weil,P.A. (1982) Specific transcription of homologous class III genes in yeast-soluble cell-free extracts. *J. Biol. Chem.*, **257**, 8432–8441.